A LITERATURE REVIEW ON THE MECHANISM OF ACTION OF SULPHUR AND NITROGEN MUSTARD

PETER J GRAY

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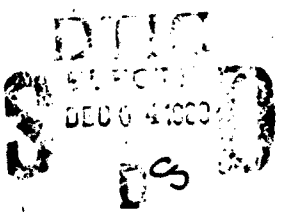

A LITERATURE REVIEW ON THE MECHANISM OF ACTION OF SULPHUR AND NITROGEN MUSTARD

Peter J. Gray

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ABSTRACT

The literature describing the mechanism of action of sulphur and nitrogen mustard is reviewed. The conclusion drawn is that the available evidence suggests that DNA is the most important molecular target of these compounds. However, the final outcome of poisoning is modulated by other factors such as DNA repair, glutathione levels and drug transport.

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CONTENTS

"CBW has come to be regarded by many people as a dirty and inhumane type of warfare, for which even most professional military men feel a very deep repugnance. A dispassionate examination of the facts, however, must surely lead to the conclusion that CBW is a clean, humane form of warfare when compared to other modern weapons systems in current use".

John Hemsley
The Soviet Biochemical Threat to NATO
Macmillan Press, 1987

A LITERATURE REVIEW ON THE MECHANISM OF ACTION OF SULPHUR AND NITROGEN MUSTARD

1. INTRODUCTION

Sulphur mustard, bis(2-chloroethyl)sulphide, (Fig. 1) was first synthesized over a hundred years ago and first used as a chemical weapon by the Germans on July 12/13 1917 near Ypres in Belgium (Robinson, 1971). This weapon is estimated to have caused approximately 400,000 casualties during the First World War (Wachtel, 1941). Since the First World War a number of compounds containing the 2-chloroethyl group have been synthesized (Robinson, 1971):

1. 2-chloroethylamines (nitrogen mustards);
2. Substituted nitrosamines;
3. higher homologues of sulphur mustard.

The nitrogen mustards (Fig. 2) have found a medical use in the treatment of Hodgkins disease (Calabresi and Parks, 1980). Sulphur mustard has recently been used extensively by Iraq against Iran (Dunn, 1987). The higher homologues of sulphur mustard (Fig. 3) are reportedly more toxic than their parent compound (Aleksandrov, 1969).

The seventy years that have elapsed since the first use of sulphur mustard as a chemical weapon have neither diminished its effectiveness nor led to a clear understanding of its mechanism of action. The recent use of sulphur mustard as a chemical weapon and the change in attitude to the use of chemical weapons as exemplified by Hemsley (1987) make it imperative that efforts be made to seek protective measures against it.

The aim of this paper is to review the literature describing the biological effects of the mustards and other alkylating agents (Fig. 4) with a view to proposing a program of research work to lead to an effective treatment of mustard poisoning.

2. CHEMISTRY

Sulphur mustard and the nitrogen mustards belong to a group of compounds which spontaneously form cyclic sulfonium or immonium ions in solution (Ross, 1962; Tilley, 1989). These reactive species are able to alkylate a number of different cellular components (Ross, 1962).

In aqueous solution, sulphur mustard undergoes nucleophilic substitution by an S_N1 mechanism via formation of a cyclic sulfonium cation (Tilley, 1989). The reaction of the nucleophile with the sulfonium cation is S_N2, however the overall rate is controlled by the ionization step and is S_N1 (Tilley, 1989). Aliphatic nitrogen mustards form stable cyclic immonium ions which react with nucleophiles by a bimolecular (S_N2) mechanism (Ross, 1962).

More extensive discussions of the chemistry of the mustards may be found in Ross (1962) and Tilley (1989).

3. PATHOLOGY

3.1 Tissue Uptake

For the purpose of this review it is sufficient to note that exposure of the skin to sulphur mustard for a few minutes is sufficient for vesication to occur although the appearance of damage may be delayed for several hours (Whitfield, 1987). The fixation and excretion of the mustards have been reviewed recently by Whitfield (1987) and will not be considered further.

3.2 Clinical Observations

In humans there is a latent period between exposure to either liquid or vapour and the onset of symptoms (Sinclair, 1945). Contamination of the skin causes erythema and blistering. Systemic effects such as nausea and vomiting may also occur (Aasted et al, 1987; Sinclair, 1945). Damage to the eyes, respiratory system and gastrointestinal tract producing symptoms which may persist for years is also possible (Aasted et al, 1987; Colardyn et al, 1986). Severely poisoned individuals exhibit bone marrow depression and may die from pulmonary infection (Colardyn et al, 1986; Dunn, 1987; Robinson, 1971; Vedder, 1925). Willems (1989) has presented an extensive clinical study of 170 sulphur mustard casualties evacuated to European hospitals from Iran between 1984 and 1986.

Similar inflammatory responses are observed in experimental animals, however, the skin does not form blisters (Bruckert, 1987; Manthei et al, 1981; Vogt et al, 1984).

4. EXPERIMENTAL OBSERVATIONS

4.1 Tissue Explants - Damage

The fur-bearing animals commonly used for laboratory studies differ from humans in their response to mustard. The damage is greater, occurs more rapidly and does not involve the blister formation characteristic of human skin (Vogt et al, 1984). In order to study the effects of mustard on human skin, Papirmeister et al (1984 a;b) used human skin grafted onto congenitally athymic nude mice.

Papirmeister et al (1984a) exposed the grafts to both liquid and vapour and observed the following:

1. a dose dependent latent period between exposure and the onset of any observable changes;
2. erythema followed by oedema;
3. hemorrhage (small);
4. ulceration;
5. separation of the epidermis from the dermis just above the basement membrane, and
6. scab formation.

Although visible blisters did not form, the response of the grafted tissue was sufficiently similar to the response of normal human tissue to suggest that the subsequent microscopic observations are relevant to human exposure.

Papirmeister et al (1984a;b) found evidence that damage begins in the basal cell layer. In addition, the earliest sign of damage appears in the nucleus. In summary, the light microscopic observations were (Papirmeister et al, 1984a);

1. minimal changes within the latency period;
2. appearance of pyknotic and karyorrhetic nuclei in the basal cells;
3. invasion of polymorphonuclear leukocytes;
4. appearance of gaps between the epidermis and dermis;
5. microabcess formation;
6. necrosis, and
7. microblister formation.

Electromicroscopic studies of the exposed grafts (Papirmeister et al, 1984b) indicated the following sequence of changes within the basal keratinocytes:

1. condensation and margination of heterochromatin and loss of euchromatin;
2. blebbing of the nuclear membrane;
3. appearance of perinuclear vacuoles;
4. swelling of the endoplasmic reticulum;
5. progressive dissociation of rosettes of free polyribosomes;
6. formation of cytoplasmic vacuoles;
7. loss of the integrity of the basal cell plasma membrane;
8. leakage of cell contents and debris into the lamina lucida;
9. disruption of the anchoring filaments of hemidesmosomes with separation of the plasma membrane from the lamina densa and accumulation of oedema fluid in the lamina lucida, and
10. infiltration of phagocytes into areas of major damage.

The main criticism of this model system, the lack of visible blistering, was ascribed to failure of the microblisters to coalesce (Papirmeister et al, 1984a). In addition, the grafts were less sensitive, by a factor of two, than human skin in situ and healed much more quickly (Papirmeister et al, 1984a).

4.2 Tissue explants - inflammatory response

Although rodent and human skins differ in structure and do not show the same type of blisters, the sequence of events observed following exposure of rodent skin is sufficiently similar to human skin to make it a reasonable model (Dannenberg et al, 1985; Vogt et al, 1984).

Dannenberg et al (1985) and Harada et al (1985;1987) have studied the inflammatory response to sulphur mustard injury using explants of rabbit skin containing both developing and healing lesions. Dannenberg et al (1984) have proposed that polymorphonuclear leukocytes accumulate in response to leukokinins released by dead cells and contaminating bacteria. Serum proteins, particularly proteinase inhibitors, enter the inflammatory lesion from the circulation and possibly play a major role in modulating the inflammatory response (Harada et al, 1985;1987).

4.3 Cell Culture and Isolated Cells

Nitrogen mustard is transported into cells by a carrier-mediated process, possibly by the choline carrier (Ritter et al, 1987; Goldenberg et al, 1970;1973). The chloroethyl group is also thought to bind to the cell surface (Linford et al, 1969).

Cultured cells exhibit many of the responses observed in tissue explants. Chromosomal aberrations develop and giant cells appear (Bernstein et al, 1987; Hartley-Asp and Hyldig-Nielsen, 1984; Roberts et al, 1968). In keratinocyte cultures preferential destruction of the basal cells occurs after exposure to sulphur mustard (Bernstein, et al; 1987). Mouse neuroblastoma cells show induction of axon growth and acetylcholinesterase, consistent with the inhibition of cell division (Lanks et al, 1975; Turnbull et al, 1973).

Treatment of cells with ^{35}S labelled sulphur mustard demonstrates alkylation of DNA, RNA and protein (Roberts et al, 1968). DNA synthesis is decreased in a dose dependent fashion (Bernstein et al, 1987; Carr and Fox, 1982; Dean and Fox, 1984; Roberts, 1978; Roberts et al, 1968). In surviving cells, inhibition of RNA and protein synthesis is either not observed (Crathorn and Roberts, 1966; Lawley and Brookes, 1965; Roberts et al, 1968) or observed only at high concentrations of mustard (Vaughan et al, 1988). However, both RNA and protein synthesis are diminished in mitochondria (Rutman et al, 1977).

Studies using synchronized cell cultures have shown alterations in the progression of cells through the cell cycle (Graham and Fox, 1983; Roberts, 1978) and variations in the sensitivity of the cells throughout the cell cycle. Cells are most sensitive if treated during G1 (Fig. 5) and least sensitive if treated during mid to late S phase (Clarkson and Mitchell, 1981; Murray and Meyn, 1986; Roberts et al, 1968). The term sensitivity refers to DNA synthesis and cytotoxicity, not alkylation which is unaffected by the position of the cell in the cell cycle (Murray and Meyn, 1986; Roberts et al, 1968). Cells treated with nitrogen mustard may be delayed in G2 after completing the S phase (Lau and Pardee, 1982).

The decrease in the rate of DNA synthesis is real, rather than a function of the disruption of cell cycle progression preventing cells from entering S phase, and is due to decreased replicon initiation and decreased DNA chain elongation in preinitiated DNA replication (Carr and Fox, 1982; Graham and Fox, 1983). The mitotic delay is then a consequence of the decrease in the rate of DNA synthesis (Roberts, 1978). The observation by Roberts et al (1968) that thymidine kinase and DNA polymerase are not affected by sulphur mustard suggests that the decrease in DNA synthesis is not due to inactivation of enzymes.

The alkylating agents vary greatly in their effects on cultured cells. Half-sulphur mustard is less toxic than sulphur mustard (Roberts et al. 1968). The monofunctional methylmethanesulphonate (MMS) is considerably less toxic than nitrogen mustard (Graham and Fox, 1983). According to Roberts (1978) ve half-sulphur mustard and 100 MMS alkylations are equivalent to one sulphur mustard alkylation. The monofunctional agents do not produce the same effects on DNA synthesis elicited by the bifunctional agents (Roberts, 1978; Roberts et al, 1968). The effects of the monofunctional agents also appear to be delayed for an additional cell cycle (Roberts, 1978). In addition, MMS does not produce the enhanced depression of DNA synthesis in sensitive cells, compared to resistant cells, produced by sulphur mustard and methylene dimethanesulphonate - both bifunctional alkylating agents (Carr and Fox, 1982).

In addition to the decrease in DNA synthesis, there is further evidence of interference in DNA metabolism. Single strand breaks have been observed in DNA extracted from cells treated with sulphur mustard (Bernstein et al, 1985;1987) and chromosomal aberrations and sister chromatid exchanges have been observed in cells treated with both sulphur and nitrogen mustard (Fox and Scott, 1980; Hartley-Asp and

Hyldig-Nielsen, 1984). Finally, both nitrogen and sulphur mustard are mutagenic, teratogenic and carcinogenic in rodents and sulphur mustard is carcinogenic in humans (Fox and Scott, 1980).

The use of cultured cells as experimental models must be treated with some caution. Different cell lines differ dramatically in their response to alkylating agents. For example, HeLa and Chinese Hamster V79-379A cells require greatly different concentrations of MMS, N-methyl-N-nitrosorea (MNU) and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) to achieve the same degree of DNA alkylation (Roberts, 1978). Murine embryonic fibroblasts and human melanoma cells also differ in the repair of DNA alkylated by nitrogen mustard (Hansson et al, 1987). Cultured cells may also change with continued passage (Erickson et al, 1980; Sognier and Hittelman, 1983). However, although the results obtained using a single cell line must be viewed with caution, comperison of strains and the correlation of the differences in the response to the mustards with other metabolic differences may yield valuable information about the mechanism of action of these compounds. Indeed, human and rodent keratinocyte cultures are becoming an important model system (Bernstein et al, 1985a;1985b).

In summary, studies using cultured cells lead to the following conclusions:

1. The cytotoxicity of the alkylating agents appears to be due to the inhibition of DNA synthesis, rather than the inhibition of cell growth;

2. There are differences in the mode of action of bifunctional and monofunctional alkylating agents and it is generally accepted that bifunctional agents are more toxic than monofunctional agents;

3. DNA damage is observed in cells treated with alkylating agents.

5. MOLECULAR MECHANISMS OF MUSTARD ACTION

The exact mechanisms by which the mustards exert their effects are not clear. However, the delay in the response of cells to exposure suggests that the toxicity is not a direct result of the interaction between the mustard and the target molecule, but involves subsequent cellular processes (Bernstein et al, 1985). The effects of sulphur and nitrogen mustard and their derivatives on a number of cellular components and processes have been studied.

5.1 Nucleic Acids

There are several points of evidence which indicate the involvement of nucleic acids in the mechanism of action of these compounds. For example, nitrogen and sulphur mustard exert their earliest visible effects on the nucleus, are mutagenic, able to inactivate transforming DNA and cause DNA damage.

5.1.1 DNA and RNA alkylation

It is now well established that sulphur and nitrogen mustard and their derivatives alkylate both DNA (Brookes and Lawley, 1960; Hemminki and Kallama, 1986; Price et al, 1968; Ross, 1962; Sack et al, 1978) and RNA (Shooter et al, 1971, 1974a, 1974b).

There are many sites at which alkylation may occur; N-1, N-3 and N-7 in adenine; N-3, N-7 and O-6 in guanine; N-1, N-3 and O-2 in cytosine and N-3 and O-4 in thymine (Fig. 6) (Lawley and Brookes, 1963; Lawley and Shah, 1972; Lawley et al, 1975; Sack et al, 1978; Shooter et al, 1974a). However, in DNA (Fig. 7), sulphur and nitrogen mustard alkylate preferentially at the guanine N-7 and adenine N-3 (Benson et al, 1988; Brookes and Lawley, 1960; Ludlum, 1984;1987; Ludlum et al, 1986; Price et al, 1968; Raikova et al, 1982;1983), with the guanine N-7 alkylation predominant. The monofunctional mustard chloroethyl ethyl sulphide (CEES) has also been shown to alkylate guanine O-6 (Ludlum, 1987; Ludlum and Papirmeister, 1986; Ludlum et al, 1986). Crosslinking of two guanine residues via the N-7 to produce N,N-bis [2-(7-guaninyl)ethyllamine or analogous products (Fig. 7) is also observed (Hemminki and Kallama, 1986).

The relative frequencies of these products in DNA exposed to sulphur or nitrogen mustard have yet to be established (Fox and Scott, 1980; Whitfield 1987). Ludlum et al. (1986) found approximately 63% guanine N-7, 11% adenine N-3 and less than 0.1% guanine O-6 alkylation after alkylation of DNA by CEES. However, approximately 16% of the adducts remain unidentified (Ludlum, 1987; Ludlum et al, 1986).

Alkylation is strongest in the interiors of sequences of contiguous guanines (Grunberg and Haseltine, 1980; Kohn et al, 1987;1988; Mattes et al, 1986; Shepherd and Sage, 1986). This preference is thought to be due to the strongly electronegative electrostatic potential which results from the clustering of G residues (Mattes et al, 1986; Pullman and Pullman, 1981). The sites responsible for interstrand cross links, 5'-GC-3', are some of the least reactive sites (Mattes et al, 1986).

Some alkylating agents are able to esterify the DNA phosphate groups (Roberts, 1978). However, although it has been proposed that sulphur mustard should be able to alkylate these groups (Roberts, 1978), no evidence of this reaction has been found (Hemminki and Kallama, 1986; Ludlum and Papirmeister, 1986).

In addition to the electrostatic potential mentioned above, the sensitivity of the alkylating agent to the strength of the nucleophile, as expressed in the Swain-Scott constant is also important in determining the result of the alkylation (Roberts, 1978; Whitfield, 1987). The location of the groups within the DNA structure may also be important. For example, the guanine O-6 and N-7 are in the DNA major groove while the adenine N-3 is in the minor groove (Roberts, 1978).

5.1.2 Consequences of DNA Alkylation

The bifunctional nature of sulphur and nitrogen mustard results in the formation of both interstrand and intrastrand crosslinks via the formation of a diguaninyl derivative (Flamm et al, 1970; Roberts, 1978). Crosslinks are also formed between DNA and protein (Dean et al, 1986; Hansson et al, 1987; Murray and Meyn, 1986; Roberts et al, 1968). In cultured cells, DNA interstrand crosslinks comprise 30-40% of the total DNA

crosslinks (excluding intrastrand crosslinks), while the remaining 30–70% is accounted for by DNA-protein crosslinks (Hansson et al, 1987). The major diguaninyl product results from the formation of intrastrand crosslinks and is estimated to be approximately 60% of the total diguaninyl product (Roberts, 1978). This is consistent with the low reactivity of the 5 -GC-3 site (see Section 5.1.1). Overall, the diguaninyl adducts account for approximately 16% of the total alkylations (Papirmeister et al, 1985). The rate of both induction and repair of the interstrand crosslinks is constant throughout the cell cycle (Clarkson and Mitchell, 1981; Murray and Meyn, 1986).

The cytotoxic action of sulphur and nitrogen mustard is not associated with the inhibition of cell growth as measured by RNA and protein synthesis (Lawley and Brookes, 1965; Roberts et al, 1968). However, the toxic effects of these compounds are associated with decreased DNA synthesis (see Section 4.3). The decreased DNA synthesis is in turn ascribed to the formation of DNA interstrand crosslinks which prevent the DNA acting as an effective template for DNA replication (Lawley and Brookes, 1965). Several studies have demonstrated a correlation between the number of mustard-induced interstrand crosslinks and cytotoxicity (Garcia et al, 1988; Hansson et al, 1987; Millar et al, 1986; Ramonas et al, 1981). This hypothesis is supported by the observation that Chinese hamster cells hypersensitive to bifunctional alkylating agents exhibit decreased ability to remove DNA crosslinks (Hoy et al, 1985). In addition, the higher toxicity of melphalan than nitrogen mustard in human melanoma cells under conditions of equal crosslink formation is related to a lower rate of removal of the crosslinks (Hansson et al, 1987). The decreased sensitivity of cells treated in G2 is then ascribed to the greater chance of the lesions being repaired before the next S phase, compared to cells treated at the transition between G1 and S (Murray and Meyn, 1986).

However, the hypothesis is by no means proven. In at least one cell line, Walker 256 rat mammary carcinoma, there is no simple correlation between cytotoxicity and cross-linking (Dean et al, 1986). In addition, the development of resistance to one bifunctional alkylating agent does not necessarily confer on the cell resistance to others (Aida and Bodell, 1987; Tan et al, 1987; Tew and Wang, 1981). Finally, sensitive and resistant cells do not necessarily differ in their ability to repair nitrogen mustard induced crosslinks (Dean et al, 1986; Tan et al, 1987). These observations do not indicate that the interstrand crosslink is not a lethal lesion, but do suggest that other factors are involved in cytotoxicity. The involvement of other factors is also suggested by the work of Tan et al (1987) who observed decreased crosslink formation in cells resistant to nitrogen mustard, but no difference in the ability of resistant and nonresistant cells to repair crosslinks.

In addition to the formation of DNA-DNA, DNA-protein and RNA-protein crosslinks, monofunctional alkylation at guanine N-7, guanine O-6 and adenine N-3 occurs (Section 5.1.1). Alkylation at guanine N-7 accounts for about 60% of the total alkylation and adenine N-3 about 16% (Papirmeister et al, 1985).

The formation of N-3 alkyladenine sensitizes the DNA to endonuclease action (Dorsey et al, 1972). N-7 alkylguanine does not produce the same sensitization (Papirmeister et al, 1985). The sensitization was attributed by Papirmeister et al (1985) to N-3-alkyladenine DNA glycosylase. Both N-3-alkyladenine and N-7-alkylguanine depurinate spontaneously (Papirmeister et al, 1985). These two alkylations may increase the rate of spontaneous apurination by a factor of 10^5 to 10^6 (Verly, 1980). The subsequent action of an apurinic endonuclease may give rise to a DNA strand break (Papirmeister et al, 1985). In E.coli, 3-methyl adenine has been identified as a major lethal lesion (Verly, 1980).

Alkylation of the guanine O-6 would be expected to alter the normal H-bond formation in DNA (Fig. 8). The formation of this product is known to cause miscoding resulting in GC to AT transitions (Gerchman and Ludlum, 1973; Lawley and Martin, 1975). The miscoding occurs because the complementary base of O-6 alkylguanine can be uracil or thymine instead of cytosine (Gerchman and Ludlum, 1973). This lesion is known to be a promutagenic lesion and procarcinogenic lesion and is also thought to be a cytotoxic lesion (Kalamegham et al, 1988; Ludlum, 1987; Newbold et al, 1980). HeLa cell mutants deficient in the ability to repair O-6-methylguanine DNA show an increased frequency of DNA strand breaks and these breaks are thought to be the cause of cell death (Kalamegham et al, 1988). Ludlum (1987) and Tan et al (1987) have proposed that the unrepaired O-6 alkylation may give rise to crosslinks. A possible mechanism for the formation of crosslinks by O-6 alkylguanine produced by CEES is shown in Fig. 9 (Ludlum, 1987).

In summary, the DNA interstrand crosslink is thought to be the lesion most likely to be responsible for cytotoxicity (Wilkinson, 1987). However, the role of the minor adducts is still unclear and may be of great importance (Ludlum, 1987). There is also evidence that factors other than the interaction of the alkylating agents with DNA modulate the response of the cell.

5.1.3 DNA Repair

Because DNA is alkylated by the mustards it is reasonable to expect that the cells DNA repair processes would influence the outcome. The most important DNA repair pathways depend on the excision of the alkylated base. The two main activities involved are provided by DNA glycosylases which cleave the base-sugar bond of the altered nucleotide, and nucleases, which incise DNA by the specific cleavage of a phosphodiester bond adjacent to a damaged residue (Lindahl, 1982). The role of DNA repair is not completely clear, although a number of components have been examined. Full discussions of the mechanism of DNA repair may be found in Lindahl (1982), Roberts (1978), Strauss (1988), Tonini (1988) and Walker (1986).

Excision repair of bulky damage by UvrABC nuclease is thought to result from detection by the enzyme system of distortions in the DNA helix (Watson et al, 1987). Frankfurt (1987) has used antibodies to detect local DNA denaturation as a result of alkylation by nitrogen mustard.

5.1.3.1 Crosslink Repair

The removal of DNA interstrand and DNA protein crosslinks has been observed by a number of workers (Clarkson and Mitchell, 1981; Dean et al, 1986; Hansson et al, 1987; Murray and Meyn, 1966). However, the exact mechanism by which the DNA interstrand crosslinks are removed is unclear. The following mechanism has been proposed (Reid and Walker, 1969; Roberts, 1978):

a) two incisions are made on one strand near a cross-link;

b) the gap is widened by a nuclease, thus exposing a single-stranded region of DNA;

c) strand exchanges between homologous duplexes insert an intact base sequence complementary to the strand still carrying the partially excised cross-linking residue;

d) when the twin helical DNA structure is restored, the remaining arm of the cross-link is excised.

As noted before (Section 5.1.2) cells sensitive to the mustards are not necessarily deficient in cross-link repair. However, cells deficient in DNA cross-link removal may be sensitive to the mustards.

Ludlum (1987) has proposed that a 7-alkyl guanine DNA glycosylase capable of recognizing the 7-alkylated guanine should be capable of repairing the cross-links.

5.1.3.2 Topoisomerase II

The enzyme topoisomerase II is involved in controlling the topological state of DNA (Wang, 1985). This enzyme is thought to alter the structure of the chromatin to allow access of mustard-induced monoadducts to repair enzymes (Tan et al. 1987).

Resistance to nitrogen mustard is associated with increased activity of DNA topoisomerase II, concomitant with decreased DNA cross-link formation (Tan et al, 1987;1988B). The increased activity is associated with increased transcription of the topoisomerase II gene. Further evidence for the involvement of this enzyme is provided by the potentiation of the lethal effect of nitrogen mustard by novobiocin - a topoisomerase II inhibitor (Tan et al, 1988a).

5.1.3.3 O-6 alkylation repair

Bacterial and mammalian cells are able to repair the O-6 methylguanine adduct (Day et al, 1980; Dean et al, 1986a; Erickson et al, 1980; Kalamegham et al, 1988; Karran et al, 1979; Robins and Cairns, 1979). The enzyme responsible for the repair, O-6-methylguanine-DNA methyltransferase, is specific for methyl groups only at the O-6 position of guanine (Lindahl, 1982).

The alkyltransferase removes ethyl groups at a much slower rate than methyl groups (Lindahl, 1982) and is totally inactive towards CEES alkylated DNA (Ludlum, 1987; Ludlum et al, 1986). Ludlum (1987) and Tan et al (1987) have proposed that the unrepaired O-6 alkylation may give rise to DNA interstrand cross-links (Section 5.1.2).

5.1.3.4 Poly(ADP-ribose)polymerase

The enzyme poly(ADP-ribose)polymerase (polyADPR-P) is indispensible for DNA excision repair (Creissen and Shall, 1982; Durkacz et al, 1980; Hayaishi and Ueda, 1982; Ueda and Hayaishi, 1985). DNA damage stimulates poly(ADP) ribosylation and a concomitant decrease in NAD^+ (Durkacz et al, 1980; Ueda and Hayaishi, 1985). Inhibition of polyADPR-P inhibits the ligation step (DNA ligase II) in DNA repair (Creissen and Shall, 1982). The NAD^+ depletion and cell death induced by the DNA alkylating agent dimethylsulphate are prevented by polyADPR-P inhibitors (Stubberfield and Cohen, 1988).

Dean and Fox (1984) and Papirmeister et al (1985) have proposed a generalized disturbance of cell metabolism involving nucleotide pool imbalance as a

consequence of intoxication by mustards. According to Papirmeister et al (1985) the sequence of events following exposure to mustard is as follows:

1. DNA alkylation;
2. spontaneous and enzymatic depurination resulting in apurinic sites and DNA breaks;
3. activation of poly(ADP-ribose)polymerase by the accumulation of DNA breaks;
4. lowering of cellular NAD^+;
5. inhibition of glycolysis and accumulation of G6P;
6. stimulation of $NADP^+$-dependent pentose phosphate cycle;
7. protease release and
8. pathological changes.

A number of the steps in the sequence have been demonstrated to occur. Depurination of alkylated bases is known to occur (Verly, 1980). Treatment of human skin grafts and leukocytes resulted in a dose-dependent decrease of up to 40% in the NAD^+ levels (Meier et al, 1984; 1987; Papirmeister et al, 1985). More importantly, in the leukocyte work, the decrease in NAD^+ only occurred in those cells (lymphocytes) containing poly(ADP-ribose)polymerase (Meier et al, 1987). Finally, inhibitors of poly(ADP-ribose)polymerase inhibited the decrease in NAD^+ (Gross et al, 1985; Papirmeister et al, 1985).

However, there are some aspects of the hypothesis which remain to be established. Although some monofunctional alkylating agents are known to stimulate the synthesis of poly(ADP-ribose) (Juarez-Salinas et al, 1979), Graham and Fox (1983) did not observe such an increase in nitrogen mustard treated cells. Secondly, although the NAD^+ decrease could be blocked by inhibitors of poly(ADP-ribose)polymerase or niacin, there was no report of a concomitant reduction in the cellular damage (Gross et al, 1985; Meier et al, 1984;1987; Papirmeister et al, 1985). The use of inhibitors of poly(ADP-ribose)polymerase is also not ideal since these compounds also inhibit the DNA repair required for the cells to recover (Meier et al, 1984). Also, as pointed out by Whitfield (1986) other inhibitors of NAD^+ synthesis such as 3-acetylpyridine and 6-aminopyridine should also be vesicants.

5.1.3.5 Depurination

As indicated in Section 5.1.2 alkylated bases may undergo spontaneous depurination. Depurination may also be enzymatically mediated. Several enzymes, DNA glycosylases, exist which are capable of breaking the sugar-base bonds in DNA and which have double stranded DNA as the preferred substrate (Lindahl, 1982). Distinct enzymes exist for the removal of 3-methyladenine and 7-methyl guanine (Lindahl, 1982). Ludlum (1987) has proposed that a 7-alkyl guanine DNA glycosylase exists which is capable of recognizing 7-ethylthioethyl deoxyguanine in CEES treated DNA and releasing it. The apurinic sites generated by the action of these enzymes should be rapidly repaired by an excision-repair process (Lindahl, 1982).

5.2 Glutathione

Glutathione (L-γ-glutamyl-L-cysteinyl-L-glycine) occurs in high concentration in cells and acts as a redox agent (Scott and Eagleson, 1988). The Orrenius theory of toxicity suggests that depletion of glutathione (GSH) is one mechanism by which compounds kill cells (see Whitfield, 1987). The involvement of GSH in the toxicity of nitrogen and sulphur mustard is still unclear. Several groups have reported increased levels of sulfhydryl groups in cells resistant to phenylalanine mustard (Begleiter et al, 1983; Suzukake et al, 1982). In addition, the formation of a diglutathione conjugate between phenylalanine mustard and GSH has been demonstrated (Dulik et al, 1986). Reduction of GSH levels by buthione sulphoximine or dietary manipulation sensitizes cells which are resistant to phenylalanine mustard (Somfai-Relle et al, 1984; Suzukake et al, 1982) while elevation of GSH by diethylmaleate increases resistance (Taylor and Brown, 1988).

The situation for nitrogen mustard is unclear. Robson et al (1987) found decreased DNA interstrand cross-link formation accompanied by elevated GSH and elevated glutathione S-transferase activity in Chinese hamster ovary cells resistant to nitrogen mustard. Conversely, Hansson et al (1988) observed increased nitrogen mustard toxicity accompanied by increased DNA interstrand cross-linking in human melanoma cells treated with nitrogen mustard and D,L-buthionine-S,R-sulfoximine. However, although Raji-NH_2 cells (resistant to nitrogen mustard) have twice the GSH present in sensitive cells, the reduction of GSH by buthione sulphoximine did not affect the cytotoxicity (Tan et al, 1987). In addition, Walker 256 cells resistant to nitrogen mustard exhibited reduced glutathione reductase activity (Dean et al, 1986).

5.3 Membrane Effects

Nitrogen mustard is known to have a number of effects on cellular functions associated with the cell membrane. Treatment of tumour cells or erythrocytes results in decreased rotational diffusion of membrane components which is ascribed to the formation of protein cross-links (Doppler et al, 1985; Grunicke et al, 1985; Levy 1965).

The use of $^{86}Rb^+$ as a K^+ congener has shown that nitrogen mustard decreases the furosemide sensitive K^+ transport, which is mediated by a $Na^+/K^+/Cl^-$ cotransporter (Baxter et al, 1982; Doppler et al, 1985; Grunicke et al, 1985; Wilcock and Hickman, 1988; Wilcock et al, 1988). It is important to note that the monofunctional mustard (2 chloroethyl)dimethylamine has no such effect (Baxter, 1982; Wilcock et al, 1988). The ouabain sensitive Na pump is only affected at relatively high concentrations of nitrogen mustard (Baxter et al, 1982; Doppler et al, 1985).

The significance of these observations is unclear. The fact that furosemide does not affect the cell proliferation indicates that the inhibition of K^+ transport by nitrogen mustard is not sufficient to explain its effects (Doppler et al, 1985; Wilcock et al, 1988). It also remains to be established whether the effect of nitrogen mustard is direct or indirect (Wilcock et al, 1988).

The trifunctional mustard tris(2-chloroethyl)amine (HN3) has been found to alkylate spectrin and cross-link it to itself and other membrane proteins in human erythrocytes (Wildenauer and Wegar, 1979). The result of the alkylation is fixation of the cell shape and protection against metabolically induced changes of shape. (Wildenauer et al, 1980). Spectrin forms the basis of the erythrocyte membrane skeleton (Bennett, 1985). No reaction between HN3 and phospholipids was detected in this experimental system (Wildenauer et al, 1980).

5.4 Cytoplasmic Effects

In addition to the effects on nucleic acids and the cell membrane, the effects on cytoplasmic components have also been studied.

Wildenauer and Weger (1979) and Fung et al (1975) have observed the alkylation of haemoglobin by nitrogen mustard. In human erythrocytes the formation of dimers was observed (Wildenauer and Weger, 1979). In isolated sickle cell haemoglobin alkylation at the $\beta 2$ histidine by nitrogen mustard was detected (Fung et al, 1975). However, sulphur mustard was not observed to alkylate haemoglobin (Fung et al, 1975).

Sulphur mustard and CEES have been observed to increase the release of lysosonal enzymes in vitro, but only at extremely high concentrations (Gross et al, 1981). In vivo, treatment of rats with nitrogen mustard also indicated that these enzymes are unlikely to be involved early in the response of the cell (Cohen, 1978).

Walker ascites carcinoma cells resistant to phenylalanine mustard exhibit a decrease in the activity of a low Km adenosine 3',5'-monophosphate phosphodiesterase (Tisdale and Phillips, 1975a). In sensitive cells, this enzyme is inhibited by treatment with chlorambucil and the concomitant increase in the level of cAMP is correlated with inhibition of cell growth (Tisdale and Phillips, 1975b).

6. PROTECTION

The pretreatment and therapy available for mustard poisoning have been reviewed recently by Whitfield (1987) and the results of that review will be presented here. The compounds considered by Whitfield are shown in Table 1 and a summary of the characteristics of potentially useful compounds in Tables 2 and 3 (from Whitfield, 1987).

Sodium thiosulphate appears to be the most effective pretreatment currently available (Fasth and Sorbo, 1973; McKinley et al, 1982; Whitfield, 1987). However, posttreatment appears to be ineffective (McKinley et al. 1982). Sodium thiosulphate is also effective in reducing the skin damage produced by nitrogen mustard in mice (Dorr et al, 1988). However a 200:1 molar excess of sodium thiosulphate was required and delaying the treatment dramatically reduced its effectiveness (Dorr et al, 1988). The use of thiosulphate combined with other drugs such as dexamethasone, cortisone, promethazine, heparin and vitamin E is effective in limiting the skin damage and increasing the survival of experimental animals exposed to sulphur mustard (Vogt et al, 1984; Vojvodic et al, 1985).

7. DISCUSSION

It is appropriate in discussing the mechanism of action of nitrogen and sulphur mustard to bear in mind the opinion of Farber (1971):

"It is eminently clear that virtually every pathologic process and disease must have a clearly defined biochemical base before we can admit to any significant understanding of that entity. Yet it is also clear that most naturally-occurring disease, be it induced by chemicals, viruses, bacteria, dietary deficiencies or other environmental alterations, is extremely complex at the level of the chemistry of the cell and may often be impossible to unravel with current knowledge. This is in part due to the existence of many interlocking regulatory control mechanisms which allow for integration and modulation of the metabolic activity of the cell".

Certainly, in the case of the mustards, the exact biochemical base has yet to be established without doubt. The observation that proliferating cells are especially sensitive, that the mustards are mutagenic and carcinogenic and that the earliest visible effects occur in the nucleus implicate DNA in the mechanism of action of these compounds. It is now well established that sulphur and nitrogen mustard alkylate DNA and produce interstrand, intrastrand and DNA-protein cross-links as well as monofunctional adducts. However, the contribution of these modifications to lethality is unclear. Opinion in the literature ranges from positive assertions that the DNA interstrand cross-link is the lethal lesion, to assigning lethality to general disturbance of DNA metabolism. Also, as Hansson et al (1987) have suggested, the position of the cross-link in the genome may be more important than the absolute number of cross-links. The monofunctional adducts are also attracting greater interest and some of these remain to be identified. The contributions to lethality of the RNA alkylations and the RNA-protein and DNA-protein cross-links have yet to be established. The transcription of DNA alkylated by nitrogen mustard produces RNA of reduced chain length (Kann and Kohn, 1972). The physiological consequences of this disturbance of RNA metabolisim have yet to be established.

In the past, alkylation of protein as a significant contribution to the lethal effects of the mustards has been discounted on theoretical grounds (Roberts, 1978; Ross, 1962). Roberts (see Farber, 1971) has stated that at the level of alkylation with sulphur mustard which inhibits DNA synthesis and cell division, the level of protein alkylation is such that only one protein molecule in 2000 (assuming a molecular weight of 100,000) will be alkylated. However, alkylation of a critical protein may well be important. For example, Wilcock et al (1988) have demonstrated on effect of nitrogen mustard on the $Na^+/K^+/Cl^-$ cotransporter, although it remains to be demonstrated whether or not this effect is secondary to the effect on DNA.

The observation that a number of DNA repair enzymes modify the lethal effects of the mustards is compelling evidence for a central role of DNA in the mechanism of action of these compounds. However, several studies have shown no difference between sensitive and resistant cell strains in their ability to repair DNA interstrand cross-links. This does not indicate that DNA damage is not the lethal lesion in these particular cells, but does indicate that other factors may be involved. The observation that cells already deficient in DNA repair are more sensitive to alkylating agents (Meyn and Murray, 1984) reinforces the central role of DNA.

Further evidence for the involvement of DNA in the lethality of the mustards is provided by the observation that caffeine enhances the lethality of nitrogen mustard (Murnane et al, 1980; Roberts and Kotsaki-Kovatsi, 1986). This enhancement is correlated with the production of double-strand breaks in the DNA (Roberts and Kotsaki-Kovatsi, 1986) and fragmentation of the nucleus (Lau and Pardee, 1982). Caffeine is

thought to reverse the inhibition of replicon initiation and thus allow the synthesis of new DNA on a damaged template (Carr and Fox, 1982). The delay in G2 is the response of the cell to the detection of the DNA damage. Tobey (1975) has proposed that a surveillance mechanism operates in G2 to ensure that damaged DNA is repaired prior to mitosis. The results of Lau and Pardee (1982) are particularly persuasive of the central role of DNA damage because the effect of caffeine was demonstrated using doses of nitrogen mustard which allowed 85% of the cells to survive in the absence of caffeine. The involvements of topoisomerase II also indicates a role for DNA in lethality.

It is also clear, as discussed above, that other factors are involved in the lethal effects of these compounds. The evidence in favour of the involvement of GSH is strong. Inhibitors of the choline transport system, which transports nitrogen mustard into the cell, also reduce the level of DNA cross-linking and protect the cells (Doppler et al, 1988). Conversley, cells with a defective choline carrier display reduced DNA cross-links and reduced sensitivity to nitrogen mustard.

The question also arises whether the mustards indiscriminately alkylate all available sites rather than preferentially alkylating nucleic acids. The study of Ritter et al (1988) is important in addressing this question. These workers found that nitrogen mustard could be recovered from Ehrlich cells, an observation inconsistent with the concept of instantaneous covalent binding of nitrogen mustard. Their uptake data suggest a two stage process whereby the nitrogen mustard is bound to a carrier and maintained is such a state that 2/3 of the unbound mustard is transferred to nondiffusible molecules or nonexchanging compartments and 1/3 is available for return to the external medium. Furthermore, vincristine decreases the effects of nitrogen mustard, suggesting that the intracellular transport of the drug is an important factor in its mechanism of action (Ritter et al, 1988). Finally, Ca^{2+} increases the uptake of nitrogen mustard but decreases its toxicity, prompting the suggestion that Ca^{2+} acts on an intracellular mechanism which directs the nitrogen mustard away from the nucleus (Ritter et al, 1988).

The fact that nitrogen mustard can exist intact within the cell suggests that it can exist in a sequestered form (Ritter et al, 1988). Rutman et al (1969) also observed the retention of functional alkylating activity bound to DNA. Their assumption that either an unreacted chloroethyl group or aziridinium ion was sequestered within the DNA structure provides some evidence for the ability of nitrogen mustard to exist in a sequestered form.

In summary, the available evidence indicates that DNA is the major target of sulphur and nitrogen mustard but other factors modulate the final effect. The exact cause of cell death is unknown. Hypotheses vary from the release of proteases (Papirmeister et al, 1985) to the ill-defined concept of 'unbalanced growth' or 'out of phase synthesis'. The consequences of many of the effects, for example RNA alkylation and the function of DNA-protein and RNA-protein cross-links, remain to be elucidated.

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TABLE 1

Possible Pretreatment and Therapy Compounds Considered by Whitfield (1987)

Dithiocarbamates

Cysteine and Cystine

Glutathione

Radioprotective Agents

Anti-arsenicals and other Thiols

Thiosulphate and its Precursors

Thiophosphonates

TABLE 2

Summary of Characteristics of Compounds Potentially useful as Pretreatments against H and HN2 Poisoning

Property	$S_2O_3^{2-}$	DMDTC	DEDTC	Disulfiram	Cysteine	GSH	WR2721
Maximum dose (mg/kg)	3.0	5	1.0	>10 mg	1.2	4.0	>0.6
Side Effects	Some nausea	Possible delayed neuropathy and others			Nausea at very high doses		Toxic
Period of effectiveness (hrs)	10 mins	1	1	24	0.5	unknown	>0.25
Route of administration	ip in large vol	ip 5 ml/kg	ip 5 ml/kg	Oral, possibly implant	ip, iv	Oral, ip	ip
Enters cells	No	Yes	Yes	Yes	Yes	Slowly	Yes
Competition factor	27,000	40,300	33,000	Unknown	1.050	<1,050	Unknown
Proven effective	Yes (N and HN2)	Promising (H)		No	Yes (HN2)	No (H)	Yes (HN2)
Available commercially	Yes	Yes	Yes	Yes	Yes	Yes	Yes

Reproduced from Whitfield (1987)

TABLE 3

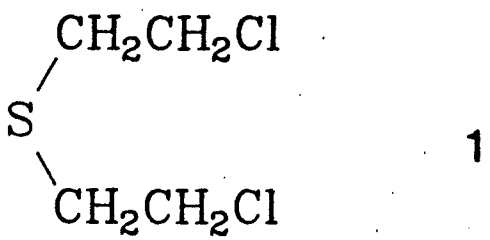
Protection indices of various sulphur-containing compounds towards murine stem cells treated in vivo with HN2

1. Maximum tolerated dose. An MTD of 10 mg/mouse corresponds to a dose of 400-500 mg/kg.
2. Protection index = $\frac{S(\text{Thiol} + HN2)}{S(\text{hn2 alone})}$

S = number of surviving femur stem cells.

Compound	MTD[1] (mg/mouse)	Protection index[2]
WR-3689	15	159
WR-2721	15	44
Aminoethylcysteine	80	27
N-acetylcysteine	8	26
Glutathione	60	22
Cysteine	8	15
2-aminoethylisothiourea (AET)	10	14
Sodium thiosulphate	50	12
2-aminoethanol	3	6.6
Cysteamine	8	3.5
WR-638	20	3.4
Disulfiram	6	1.1
Ethylamine	1	1.0
2-mercaptoethanol	3	0.6
Dimethylsulfoxide	250	0.49

Reproduced from Whitfield (1987)



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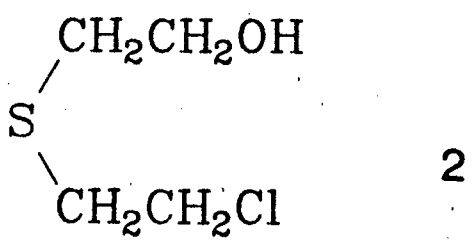
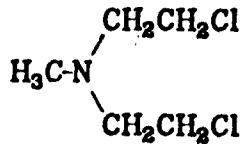


2

Figure 1 Sulphur mustard (1) and half mustard (2).



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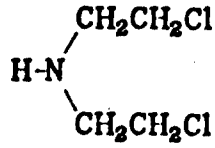

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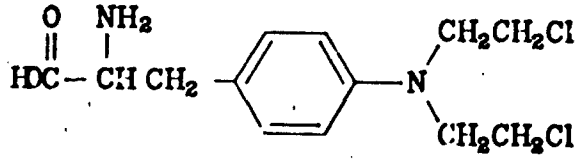

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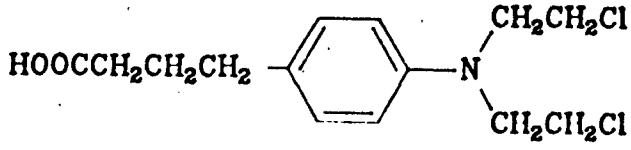
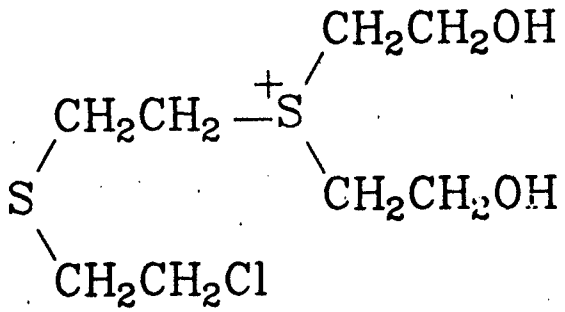


4

Figure 2 Nitrogen mustard and derivatives.
1. *Nitrogen mustard (HN2)*
2. *Normitrogen mustard*
3. *Phenylalanine mustard*
4. *Chlorambucil*



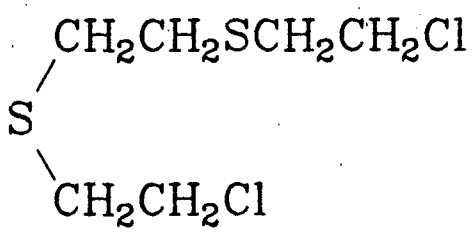
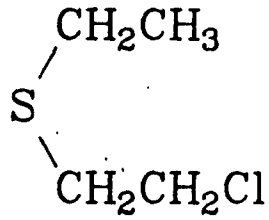


Figure 3 Higher homologues of sulphur mustard.



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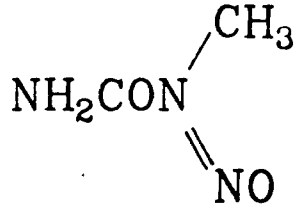

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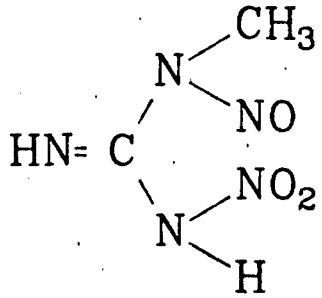

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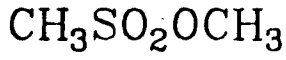


4

Figure 4 *Additional alkylating agents:*
1. *Chloroethyl ethyl sulphide*
2. *N-methyl-N-nitrosourea*
3. *N-methyl-N'nitro-N-nitrosoguanidine*
4. *methyl methane sulphonate.*

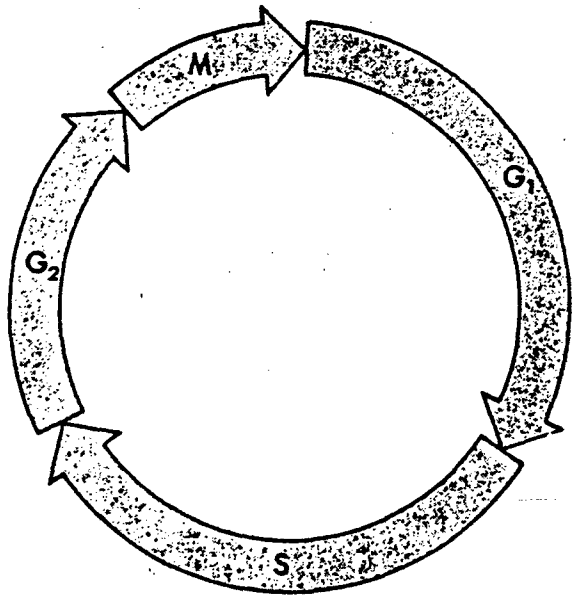
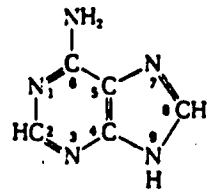


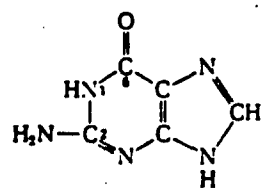
Figure 5 Phases in the life cycle of a cell. M1 is the mitotic phase and S the DNA synthetic phase. The two gap phases are G1 (pre-DNA synthesis) and G2 (period between DNA synthesis and mitosis). The relative lengths of the various phases differ between cell types.

The major purines

Adenine

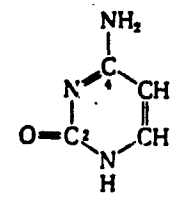


Guanine

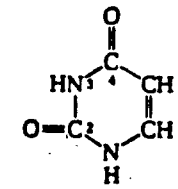


The major pyrimidines

Cytosine



Uracil



Thymine

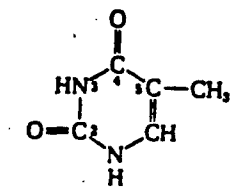


Figure 6 The major purines and pyrimidines.

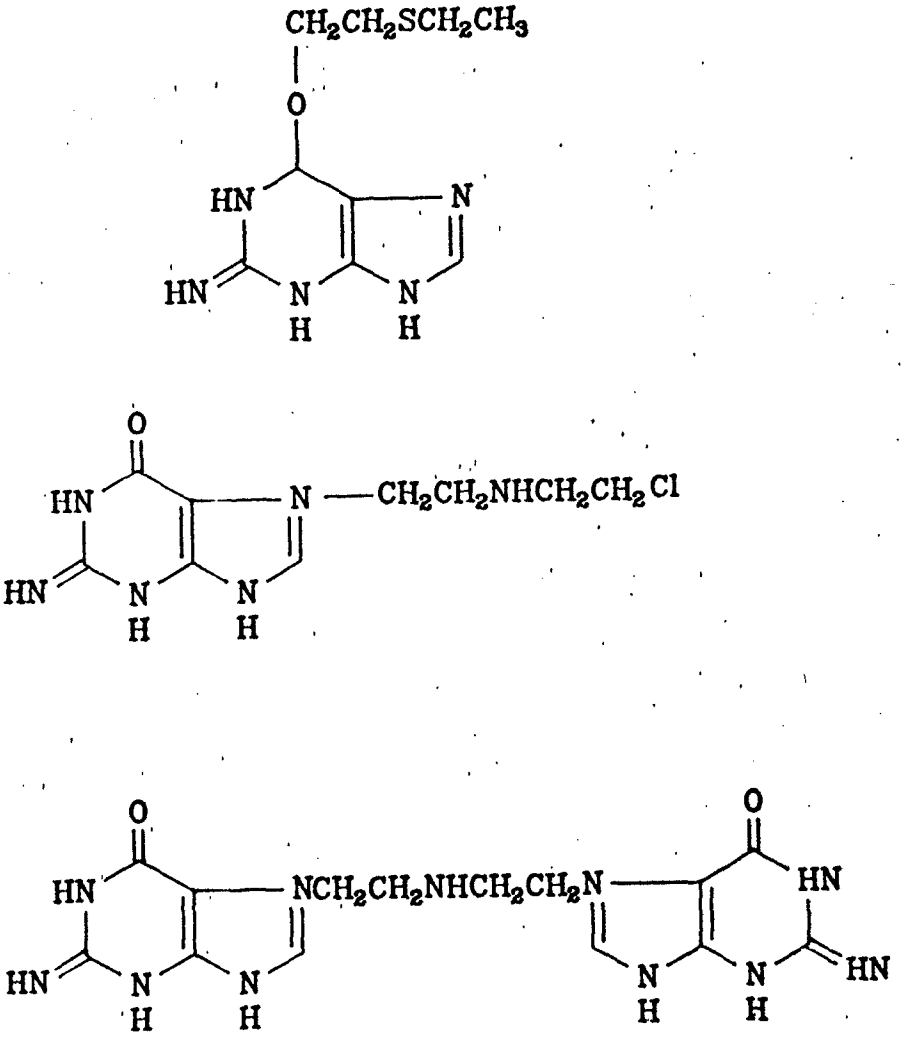


Figure 7 *Products from the alkylation of DNA by nitrogen mustard and CEES:*
Upper - O^6 ethylthioethyl guanine
Centre - 7 ethyl thioethyl guanine
Lower - N, N-bis [2-(7-guaninyl)-ethyl]amine.

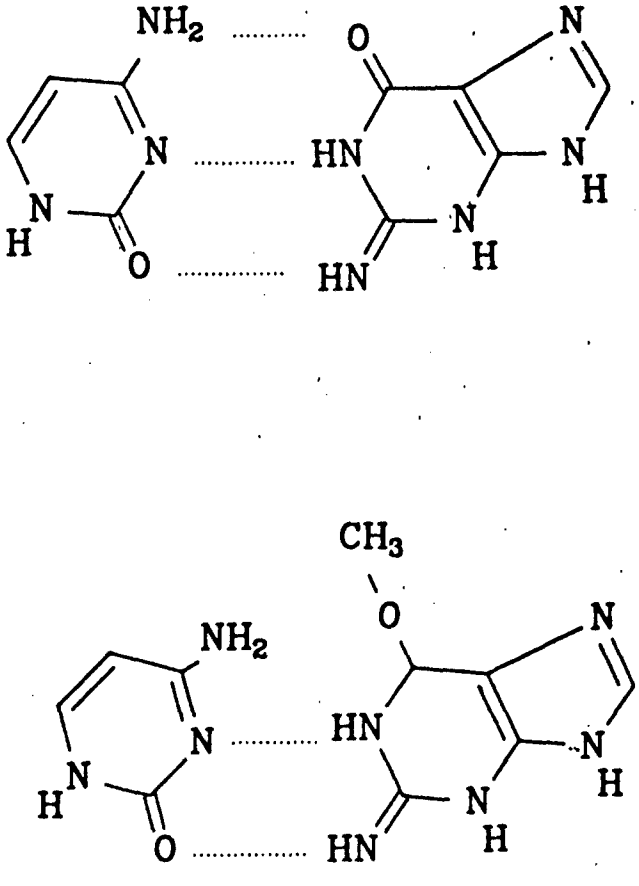


Figure 8 Altered H-bonding by formation of O^6 alkylguanine. Guanine normally pairs with cytosine (top). Methylation disrupts the normal H-bonding (bottom) and allows pairing with thymine.

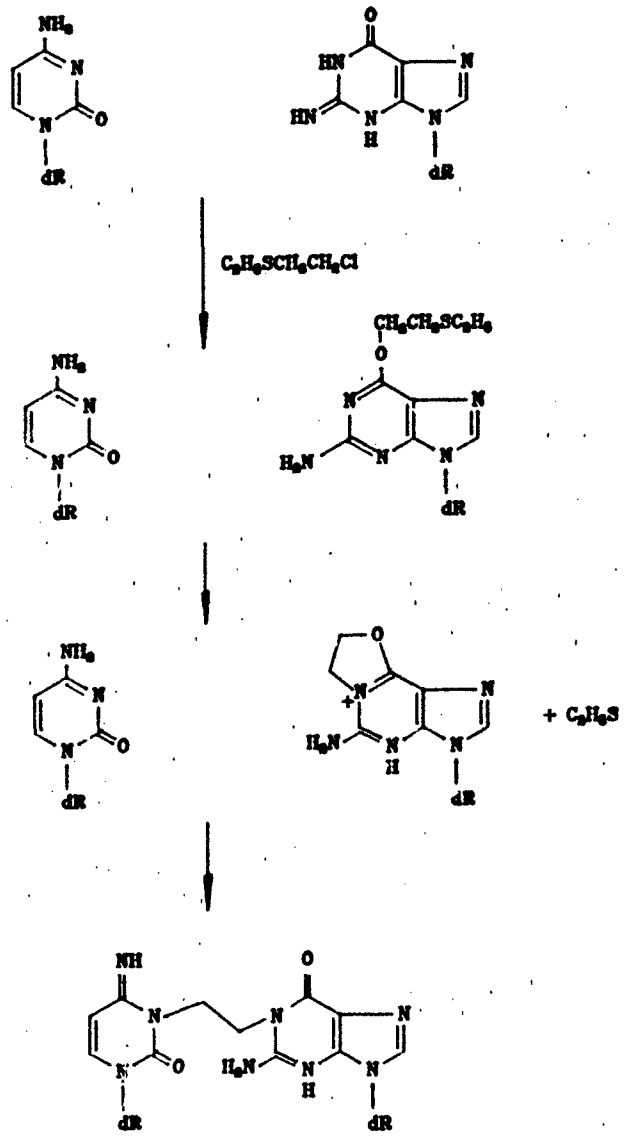


Figure 9 Mechanism proposed for the formation of DNA crosslinks by CEES. (From Ludlum, 1987).

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Nitrogen Mustards
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ABSTRACT

The literature describing the mechanism of action of sulphur and nitrogen mustard is reviewed. The conclusion drawn is that the available evidence suggests that DNA is the most important molecular target of these compounds. However, the final outcome of poisoning is modulated by other factors such as DNA repair, glutathione levels and drug transport.